Cell, Vol. 84, 73–82, January 12, 1996, Copyright ©1996 by Cell Press

Cdk2 Kinase Is Required for Entry into Mitosis as a Positive Regulator of Cdc2–Cyclin B Kinase Activity

Thomas M. Guadagno and John W. Newport
Department of Biology
University of California, San Diego
La Jolla, California 92093-0347

Summary

In higher eukaryotes, Cdk2 kinase plays an essential role in regulating the G1–S transition. Here, we use cycling Xenopus egg extracts to examine the requirement of Cdk2 kinase on progression into mitosis. Interestingly, when Cdk2 kinase activity is inhibited by the Cdk-specific inhibitor, $p21^{Cip1}$, a block to mitosis occurs, and inactive Cdc2–cyclin B accumulates. This block occurs in the absence of nuclei and is not due to direct inhibition of Cdc2 by Cip. Importantly, this block to mitosis is reversible by restoring Cdk2–cyclin E kinase activity to a Cip-treated cycling extract. Moreover, immunodepletion of Cdk2 from interphase extracts prevents activation of Cdc2 upon the addition of exogenous cyclin B. Thus, our data show that Cdk2 kinase is a positive regulator of Cdc2–cyclin B complexes and establish a link between Cdk2 kinase and cell cycle progression into mitosis.

Introduction

In higher eukaryotes, cell proliferation is controlled by a family of cyclin-dependent kinases (Cdks). At the G2/M transition, mitosis is initiated by a Cdk–cyclin complex (maturation-promoting factor [MPF]) consisting of the Cdc2 protein kinase and a B-type cyclin (Dunphy et al., 1988; Gautier et al., 1988; Draetta et al., 1989). Activation of MPF is controlled both by the accumulation of cyclin B and by three phosphorylation sites on the Cdc2 subunit. During the embryonic cell cycles of Xenopus, mitotic cyclins A1, B1, and B2 are synthesized during interphase and form complexes with Cdc2 (Minshull et al., 1989, 1990). A stimulatory kinase known as Cdk-activating kinase (CAK) phosphorylates Cdc2 on the Thr-161 residue, which is essential for Cdc2 catalytic activity (Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993). Prior to MPF activation, Cdc2–cyclin B is held inactive by phosphorylations at Tyr-15 and Thr-14 residues of Cdc2 (Solomon et al., 1990). The inhibitory kinase, Wee1, phosphorylates Cdc2 specifically on Tyr-15 (Russell and Nurse, 1987; Featherstone and Russell, 1991), and a distinct membrane-associated kinase is thought to phosphorylate Thr-14 (Kornbluth et al., 1994; Atherton-Fessler et al., 1994; Mueller et al., 1995).

At the onset of mitosis, Cdc25 phosphatase activates MPF by dephosphorylating Cdc2 on Tyr-15 and Thr-14 (Dunphy and Kumagai, 1991; Gautier et al., 1991). One of the kinases that activates Cdc25, at least in vitro, is the Cdc2–cyclin B complex, a target of Cdc25 (Hoffmann et al., 1993; Izumi and Maller, 1993). This has suggested the existence of a positive feedback loop between Cdc2 and Cdc25, which might explain the abrupt amplification of MPF activity observed upon entry into mitosis (Solomon et al., 1990). Because CAK activity appears to be constant throughout the cell cycle (Fisher and Morgan, 1994; Matsuoka et al., 1994), activation of Cdc2 kinase at mitosis is likely regulated by a decrease in the Thr-14/Tyr-15 kinases that negatively phosphorylate Cdc2 or by an increase in Cdc25 phosphatase activity, which positively activates Cdc2 kinase (Smythe and Newport, 1992). However, little is known about possible upstream regulators that might modulate changes in Wee1 kinase and Cdc25 phosphatase activities.

Unlike yeast, in which a single Cdc2 protein controls both the G1–S and G2–M transitions, higher eukaryotes use distinct Cdks to coordinate cell cycle progression throughout the G1 and S phases (for reviews see Sherr, 1994; Hunter and Pines, 1994). A number of key observations have shown that Cdk2 kinase is necessary for entry into S phase (for review see Heichman and Roberts, 1994). In particular, immunodepletion of Cdk2 kinase (Fang and Newport, 1991) or inhibition of its kinase activity by the Cdk inhibitor protein, $p21^{Cip}$ (Strausfeld et al., 1994; Yan and Newport, 1995), blocks DNA synthesis in Xenopus egg extracts. Additionally, a G1 phase arrest is observed when a kinase-defective mutant of Cdk2 is expressed transiently in somatic cells (van der Heuvel and Harlow, 1993). In somatic cells, Cdk2 associates with cyclins A and E (Koff et al., 1991; Lew et al., 1991; Tsai et al., 1991). During the somatic cell cycle, Cdk2 kinase activity is first detected at the late G1 phase of the cell cycle and is strongest during S and G2 phases (Pagano et al., 1992, 1993; Rosenblatt et al., 1992; Tsai et al., 1993). In contrast with somatic cells, Cdk2 kinase is primarily bound to cyclin E during the rapid, early embryonic cell divisions in Xenopus eggs, and this complex remains active throughout the cell cycle (Fang and Newport, 1991; Rempel et al., 1995; Howe and Newport, submitted). Despite having a role in promoting entry into S phase, the presence of active Cdk2–cyclin complexes throughout S and M phases of the embryonic cell cycle suggests that it might regulate other cell cycle–related events.

Similar to budding yeast, the commitment to cell division in most vertebrate cells is regulated by a START-like control or "restriction point" in late G1 phase of the cell cycle (Pardee, 1989; Sherr, 1994). This has suggested the existence of a signaling pathway that is initiated at the commitment step of cell division and promotes cell cycle progression throughout S phase and mitosis. Conceivably, this signaling pathway might involve the sequential activation of distinct Cdk complexes from G1–S to mitosis. Precedence for a regulatory network between distinct Cdk–cyclin protein kinases comes from yeast. In budding yeast, late G1 expression of Cln–Cdc28 kinases is necessary for turning off the Clb cyclin degradative machinery (Amon et al., 1994) and subsequently allowing the expression and activation of Clb–Cdc28 complexes, which are critical for DNA replication and entry into mitosis (Schwob et al., 1994). Indeed, in higher eukaryotes, Cdk2 kinase is

a positive cell cycle regulator of S phase and, therefore, may regulate the activity of downstream Cdk–cyclin complexes. However, a role for Cdk2 kinase in positively regulating downstream Cdk–cyclin complexes is difficult to demonstrate in somatic cells owing to the existence of multiple negative checkpoint controls that coordinate the cell cycle (reviewed by Nurse, 1994). Included in these checkpoint controls are the attainment of an adequate cell size, the completion of DNA replication, and the repair of damaged DNA, all of which are necessary before a cell can initiate mitosis.

To circumvent the effects of checkpoint controls on cell cycle progression, we chose to utilize the cell-free system of Xenopus egg extracts. Extracts prepared from unfertilized Xenopus eggs can be programmed to carry out cell cycles in vitro that closely mimic the cell cycle events observed in vivo (Murray and Kirschner, 1989). Importantly, the oscillation of extracts between S phase and mitosis can occur in the absence of growth, DNA replication, and DNA repair. As such, by using these extracts we could ask whether Cdk2 kinase is required for controlling entry into mitosis. The data presented in this report provide direct biochemical evidence for an essential role of Cdk2 kinase in positively regulating Cdc2–cyclin B complexes during the cell cycle.

Results

Cip Inhibits Initiation of Mitosis in Cycling Egg Extracts

Extracts made from unfertilized eggs in the presence of EGTA are arrested in metaphase (cytostatic factor [CSF]-arrested extracts). Addition of Ca^{2+} to such extracts causes cyclin B to be degraded, and the extract enters interphase of the cell cycle. The reaccumulation of mitotic cyclins during interphase typically causes the extract to reenter mitosis after 80–95 min (Minshull et al., 1989; Murray et al., 1989). We have used such extracts and the 21 kDa Cdk2 kinase inhibitor, $p21^{Cip1}$, to determine whether Cdk2 activity is essential for initiation of mitosis. To do this, 50 μl aliquots of CSF extract were preincubated with 125 nM glutathione S-transferase (GST) without Cip or with GST–Cip for 15 min at 4°C to inhibit all Cdk2 kinase activity (see Experimental Procedures). At time zero, the CSF-arrested extracts were activated by the addition of 0.4 mM Ca^{2+} and allowed to incubate at room temperature in the presence of sperm chromatin (500 nuclei per microliter). In control extracts preincubated with GST protein alone, nuclei formed around added sperm chromatin, and by 60 min the chromatin became very decondensed (Figure 1A). In GST–Cip-treated extracts, nuclei also formed, but the DNA consistently remained more condensed (Figure 1C). At 90 min, the control extract entered mitosis, as evidenced by breakdown of nuclei and accumulation of condensed chromosomes (Figure 1B). By contrast, using this same criteria, GST–Cip-treated extracts failed to enter mitosis over the full 2.5 hr period of the experiment (Figure 1D). Therefore, quite surprisingly, these observations show that inhibition of Cdk2 kinase activity in cycling egg extracts blocks the onset of mitosis.

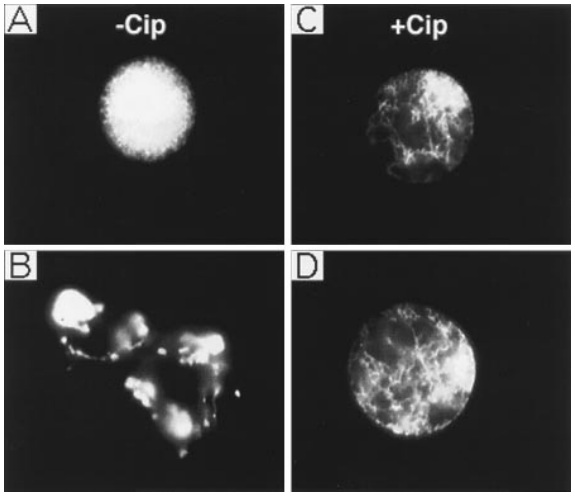

Figure 1. Cip Blocks Mitotic Breakdown of Nuclear Envelope in Ca^{2+}-Activated CSF Extracts

Cell cycle progression of Ca^{2+}-activated CSF extracts was monitored visually every 10–15 min by fluorescence microscopy of fixed nuclei stained with bisbenzimide. In control extract (minus Cip), nuclear membrane breakdown and chromosome condensation occurred at 90 min (B). Cip-treated extracts remained in interphase (intact nuclei) throughout 2.5 hrs (C and D). Photographs were taken at 60 min (A and C, interphase), 90 min (B, mitosis), and 150 min (D, interphase).

Inhibition of Mitosis by Cip Is Not Due to the Presence of Unreplicated DNA

During S phase of the cell cycle, ongoing DNA synthesis activates a checkpoint pathway that inhibits Cdc2 kinase activity to prevent premature mitosis (Hartwell and Weinert, 1989; Dasso and Newport, 1990; Enoch et al., 1992). Because Cdk2 is essential for DNA replication, the interphase arrest observed in the presence of Cip may be due to the activation of this replication-dependent feedback pathway. If this were the case, then we would expect that control and Cip-treated extracts lacking DNA would enter mitosis at similar times. To investigate this possibility, we set up experiments similar to those above in the absence of sperm chromatin and monitored entry into mitosis. The onset of mitosis was followed by using two reliable biochemical indicators for this event. Specifically, both Cdc2 kinase and Cdc25 phosphatase are subject to well-characterized changes in phosphorylation at mitosis. Cdc2 is dephosphorylated at both Thr-14 and Tyr-15 at mitosis (Gould and Nurse, 1989; Solomon et al., 1990), and Cdc25 is phosphorylated at many sites at this time (Kumagai and Dunphy, 1992; Izumi et al., 1992). These mitotic alterations cause changes in the electrophoretic mobility of these two proteins, such that Cdc2 runs faster and Cdc25 runs more slowly on SDS–polyacrylamide gels at mitosis. Thus, these mitosis-dependent changes in the electrophoretic mobility of Cdc2 kinase and Cdc25 phosphatase are a reliable indicator for determining when the cycling extracts enter mitosis.

In the control extract, as expected, a fraction of Cdc2 accumulated as a phosphorylated (Thr-14–Tyr-15), slowly migrating form during interphase and then abruptly became dephosphorylated between 80 and 100

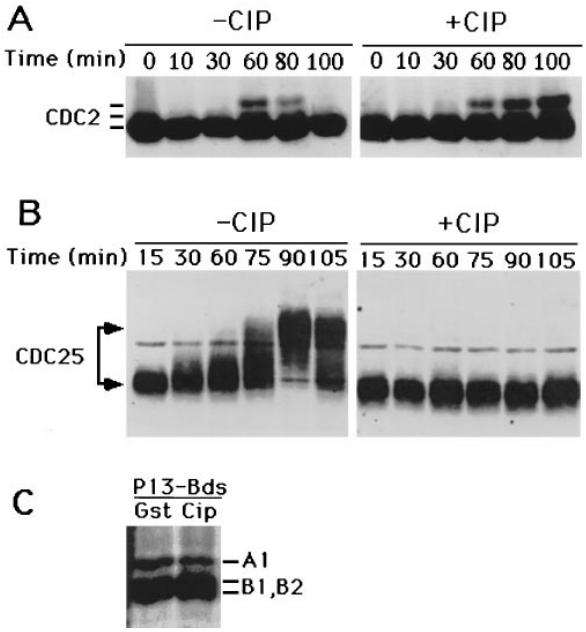

Figure 2. Effects of Cip on Cdc2 and Cdc25 Activity

(A) Accumulation of phosphorylated forms of Cdc2 in Cip-treated extracts. Aliquots from Ca^{2+}-activated extracts incubated in the absence (minus CIP) or presence of 125 nM GST–Cip were withdrawn at the indicated times and resolved by SDS–PAGE. Mobility shifts due to phosphorylation of Cdc2 were detected by Western blot analysis. The two upper bands (top two notches on the left panel) represent the inhibitory phosphorylations of Cdc2 on Thr-14, Tyr-15, or both.

(B) Activation of Cdc25 phosphatase is blocked in cycling extracts treated with Cip. A similar time course experiment as above was performed to assay for the mitotic activation of Cdc25 phosphatase. Western blot analysis was performed with an affinity-purified Cdc25 polyclonal antibody. The lower band (indicated by lower arrow) represents the unphosphorylated form of Cdc25. Mitotically active Cdc25 (upper arrow) migrates as a slower-migrating form owing to hyperphosphorylation (see minus CIP panel at 90 and 105 min).

(C) Accumulation and association of mitotic cyclins with Cdc2 is not affected by Cip. Ca^{2+}-activated extracts in the presence of [^{35}S]methionine were incubated for 75 min with 125 nM GST or GST–Cip protein. Parallel samples containing sperm nuclei allowed visual confirmation that the cycling extracts were in interphase at 75 min. Cdc2–cyclin complexes were isolated on p13–Sepharose beads, boiled in SDS-containing sample buffer, and resolved by SDS–PAGE. The ^{35}S-labeled mitotic cyclins A1, B1, and B2 were visualized by autoradiography.

min (Figure 2A, minus Cip), demonstrating that the extract had entered mitosis at this time. In a similar control extract, Cdc25 became partially phosphorylated between 30 and 75 min and abruptly hyperphosphorylated by 90–105 min, as evidenced by its shift to a slower-migrating form (Figure 2B, minus Cip). By contrast, neither of these two changes occurred in Cip-treated extracts; Cdc2 continued to accumulate in the phosphorylated, inactive, slow-mobility form (Figure 2A, plus Cip), and Cdc25 remained dephosphorylated (Figure 2B, plus Cip) throughout the experiment. Taken together, these experiments demonstrate that inhibition of Cdk2 kinase activity blocks the initiation of mitosis in the absence of nuclei. Therefore, the failure of Cip-treated extracts to enter mitosis is independent of the feedback pathway generated by ongoing DNA replication.

Cip Does Not Inhibit Mitosis by Inhibiting Cdc2

If Cip inhibited the activity of Cdc2 kinase, this would readily explain its ability to inhibit the initiation of mitosis. Such inhibition could occur because Cip blocks the synthesis of mitotic cyclins or, alternatively, because Cip inhibits Cdc2 directly by binding to it. To address these possibilities, we performed the following experiments. Activated CSF extracts, containing either GST–Cip or GST alone, were labeled with [^{35}S]methionine, and the Cdc2–cyclin complexes were then precipitated with p13–Sepharose. Following SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the p13–Sepharose precipitates, Cdc2-associated cyclins were quantitated. The results showed that the amount of mitotic cyclins A1, B1, and B2 synthesized and associated with Cdc2 did not differ between control and Cip-treated extracts (Figure 2C). Therefore, Cip does not appear to inhibit either cyclin synthesis or its association with Cdc2.

In another experiment, Cip was added to mitotic CSF-arrested extracts at a concentration sufficient to inhibit completely Cdk2 kinase activity and block initiation of mitosis. If Cip inhibited Cdc2 directly, such treatment should cause the extract to enter interphase. However, we found that the extracts remained arrested in mitosis in the presence or absence of Cip. This was shown both visually and biochemically. Specifically, in extracts to which sperm chromatin was added, the DNA remained condensed and did not form nuclei (Figures 3A and 3C). Consistent with this, the H1 kinase activity of Cdc2 in CSF-arrested extracts is not reduced in the presence of Cip (Figure 3E, minus Ca^{2+}). Moreover, when Ca^{2+} was added to these extracts, they entered interphase normally, as determined by the formation of nuclei (Figures 3B and 3D) and by inactivation of Cdc2 kinase (Figure 3E, plus Ca^{2+}). Thus, although Cip inhibits the initiation of mitosis, once an extract is in mitosis addition of Cip neither inhibits the mitotic state nor blocks the ability of the extract to exit mitosis. We conclude that Cip does not inhibit Cdc2–cyclin B kinase activity directly at concentrations that block entry into mitosis.

Cip Binds to Cdk Complexes Containing Cyclins E and A, but Not B-Type Cyclins

The experiments above strongly indicate that Cip does not interact with active Cdc2–cyclin B complexes. To determine which cyclin complexes do interact with Cip, GST–Cip fusion protein was added to cycling extracts in interphase of the cell cycle, and complexes associated with the fusion protein were then precipitated on glutathione–Sepharose beads. The composition of the precipitated proteins was analyzed on Western blots using antibodies to Cdk2 and different cyclin proteins. Identical extracts were also precipitated with p13–Sepharose beads. Because p13 quantitatively binds both Cdc2 and Cdk2, this control provided a basis for determining how efficiently a particular Cdk–cyclin complex bound to GST–Cip. As expected, the results from this experiment (Figure 4A) showed that Cdk2–cyclin E complexes bound tightly to Cip. Similarly, cyclin A1, which is primarily associated with Cdc2 in eggs (Minshull et al., 1989; Rempel et al., 1995), was also selectively

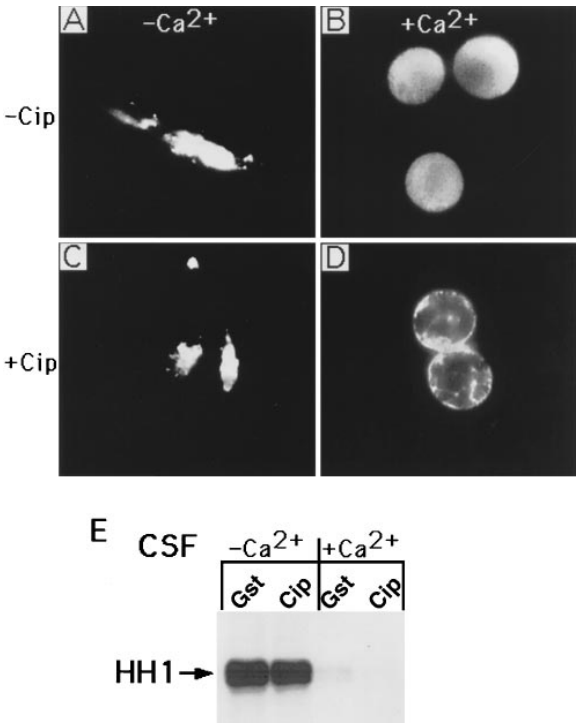

Figure 3. CSF-Arrested Extracts Retain Mitotic Cdc2–Cyclin B Activity in the Presence of Cip

Sperm chromatin (250 nuclei per microliter) was added to CSF extracts in the presence or absence of 300 nM Cip protein. The CSF extracts were incubated for 45 min at 22°C.

(A–D) Following fixation and staining, sperm chromatin was visualized by fluorescence microscopy. CSF extracts without Cip (A) and with Cip (C) show similar sperm chromatin condensation. Ca^{2+}-activated CSF extracts exit mitosis and form intact nuclei in the absence (B) or presence (D) of Cip protein.

(E) CSF or Ca^{2+}-activated CSF extracts were incubated for 30 min in the absence or presence of 300 nM Cip protein. We assayed 2 μl aliquots for H1 kinase activity. Note that addition of Cip protein (up to 300 nM) directly into an H1 kinase assay mix did not inhibit Cdc2 activity.

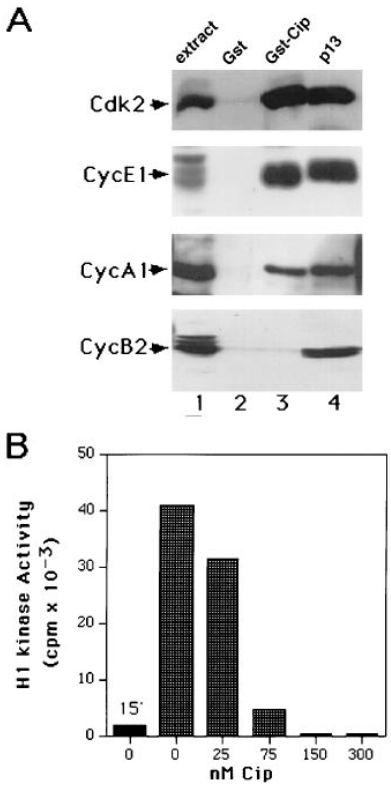

Figure 4. Characterization of Cip Binding to Cdc2 and Cdk2 Complexes

(A) 125 nM GST or GST–Cip protein was incubated in Ca^{2+}-activated extracts for 75 min. Associated complexes were isolated on glutathione–Sepharose beads and analyzed by Western blot analysis with specific antibodies for Cdk2, cyclin E1 (CycE1), cyclin A1 (CycA1), and cyclin B2 (CycB2). Lane 1, 1 μl of interphase extract; lanes 2–4, GST, GST–Cip, and p13 complexes from 25 μl of interphase extract.

(B) Cip inhibits the activation of Cdc2–cyclin B kinase complexes in the absence of cyclin A1. Interphase egg extracts prepared in the presence of cycloheximide were mixed with increasing concentrations of Cip protein (0–300 nM final concentration). Western blot analysis showed that cyclin A1 protein was undetectable in cycloheximide-treated extracts. Nondegradable GST–cyclin B protein (35–70 nM final concentration) was incubated with each sample, and Cdc2 kinase activation was determined by histone H1 kinase assays. Closed bars, 15 min; stippled bars 75 min. Control samples containing sperm nuclei entered mitosis at 50 min (0–25 nM Cip), while nuclei of Cip-treated extracts (75–300 nM) remained intact (interphase arrested) for at least 90 min.

precipitated. By contrast, cyclin B2 was not precipitated by glutathione beads, but was by p13–Sepharose beads. In an experiment similar to that above, ^{35}S labeling of newly synthesized proteins showed that cyclin A1 was detected in complexes bound to GST–Cip, but not cyclins B1 and B2 (data not shown). These results show that in Xenopus egg extracts Cip associates tightly with both Cdc2–cyclin A and Cdk2–cyclin E complexes, but not with Cdc2–cyclin B complexes.

The Inhibition of Mitosis by Cip Is Independent of Cyclin A

We have shown above that Cip blocks the initiation of mitosis in cycling extracts. Further, we have shown that Cip does not bind strongly to the primary mitotic regulator in eggs or Cdc2–cyclin B, nor does it inhibit the kinase activity of this complex once it is active. With regard to how Cip inhibits mitosis, our data suggest two possible targets, Cdc2–cyclin A and Cdk2–cyclin E. To distinguish between these two possibilities, we have investigated whether Cip blocks initiation of mitosis in extracts that contain Cdk2–cyclin E but lack Cdc2–cyclin A. When eggs are lysed in the presence of the protein synthesis inhibitor cycloheximide, cyclins A1, B1, and B2 are degraded. Because the extract cannot resynthesize new mitotic cyclins, it remains arrested in interphase. The level of Cdk2–cyclin E kinase in these extracts remains high (Fang and Newport, 1991; Rempel et al., 1995; Howe and Newport, submitted). Initiation of mitosis in such extracts will occur if exogenous cyclin B is added (Minshull et al., 1989; Murray et al., 1989). Therefore, such extracts represent an excellent system for investigating the regulation of mitotic initiation in the absence of cyclin A.

Using such cycloheximide-arrested extracts, we have determined whether mitosis, which normally occurs after the addition of a threshold concentration of cyclin

B protein, is inhibited in the presence of Cip. To do this, we first preincubated cycloheximide-arrested extracts with different concentrations of Cip (0–300 nM). A nondegradable GST–cyclin B fusion protein (35 nM) was subsequently added to each sample. We monitored entry into mitosis in each sample by visually examining of the state of nuclei and measuring Cdc2 kinase levels 75 min later. In the absence of Cip, Cdc2-dependent H1 kinase levels increased 20-fold by 75 min when compared with 15 min (Figure 4B). However, in the presence of Cip (75–300 nM), Cdc2 kinase activation was inhibited by at least 90% relative to the control. This correlates with the minimal concentration of Cip protein (75 nM) required to inhibit endogenous Cdk2 kinase activity completely. Consistent with the H1 kinase measurements, we observed that, while the nuclei in control extracts underwent nuclear envelope breakdown and chromosome condensation at 50 min, the nuclei in Cip-treated extracts remained intact for at least 90 min. These observations strongly suggest that the block to the initiation of mitosis observed in the presence of Cip protein is independent of Cdc2–cyclin A complexes. Interestingly, it should be noted that when very high levels of GST–cyclin B protein (240 nM) were added to cycloheximide-arrested extracts containing Cip, Cdc2 kinase activity increased rapidly, and the extract entered mitosis. This indicates that Cip blocks the initial activation step of Cdc2–cyclin B, but does not inhibit the autocatalytic loop, which causes rapid amplification of the bulk of Cdc2–cyclin B activity following the activation of small amounts of Cdc2 kinase (Solomon et al., 1990; Hoffmann et al., 1993).

The results presented above strongly suggest that the inhibition of mitosis in Cip-treated cycling extracts is a direct result of the inhibitory effect of Cip on the catalytic activity of Cdk2–cyclin E complexes. This implies that under normal conditions, in the absence of Cip, Cdk2–cyclin E is a positive regulator essential for the initiation of mitosis. Although this proposal is surprising given the demonstrated role of Cdk2–cyclin E in initiation of DNA replication (Fang and Newport, 1991; Yan and Newport, 1995; Jackson et al., 1995), it does make two strong predictions that can be tested directly. First, the proposal predicts that addition of excess Cdk2–cyclin E complexes to Cip-treated cycling extracts should reverse the Cip arrest and allow the extract to enter mitosis. Second, immunodepletion of Cdk2–cyclin E complexes from an extract should, like Cip treatment, prevent the extract from entering mitosis. The results of experiments designed to test these two predictions are presented below.

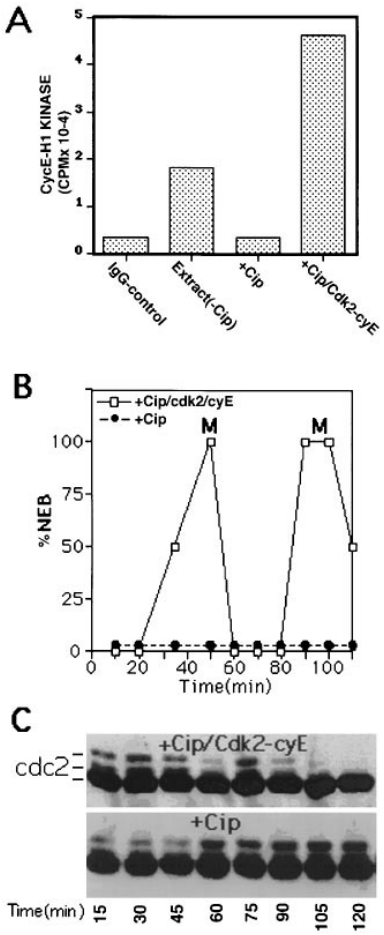

Figure 5. Addition of Cdk2–Cyclin E Reverses the Block to Mitosis by Cip

(A) Assay of cyclin E–associated H1 kinase activity.

(B) The assembly and disassembly of nuclear envelope was monitored at 10 min intervals by phase-contrast microscopy and expressed as percent nuclear envelope breakdown (NEB). Cip-treated extracts failed to enter mitosis. Cip-treated extracts in which Cdk2–cyclin E complexes were added restored Cdk2–cyclin E kinase activity and cycled through mitosis (M) at 50 min and again at 100 min. The positive control extract cycled into mitosis at 50 min and again at 90 min (data not shown).

(C) Western blot analysis of the phosphorylated state of Cdc2. The upper two bands represent the inactive, phosphorylated forms of Cdc2 kinase. Note that H1 kinase assays of Cdc2 activity correlated with the results in Figures 5B and 5C.

The Block to Mitosis by Cip Is Reversible by Restoring Cdk2–Cyclin E Kinase Activity

GST fusions of Xenopus Cdk2 and cyclin E proteins were purified from bacterial lysates to test whether addition of these proteins to cycling extracts could reverse a Cip-induced arrest. For this experiment, an extract that undergoes multiple cell cycle oscillations was prepared (Murray and Kirschner, 1989) and split into three equal aliquots. No further additions were made to one aliquot (control), 100 nM Cip was added to the remaining two aliquots, and 250 nM of both GST–Cdk2 and GST–cyclin E was added to one of these Cip-containing aliquots. The kinase activity of Cdk2–cyclin E present in each aliquot was measured by immunoprecipitating a portion of the sample with cyclin E antibodies. The cyclin E–associated H1 kinase activity in extracts treated with 100 nM Cip alone was negligible when compared with background (compare IgG control with plus Cip in Figure 5A). By contrast, the Cdk2–cyclin E kinase activity in extracts containing both Cip and GST–Cdk2–cyclin E was 2.5 times higher than the endogenous levels (Figure 5A, compare control extract with plus Cip/Cdk2–cyclin E). This demonstrates that the added Cdk2–cyclin E is both active and in excess over added Cip.

During the experiment, aliquots were withdrawn every 10–15 min over a 2 hr period and analyzed for nuclear envelope breakdown, H1 kinase activity, and shifts in Cdc2 protein mobility resulting from cell cycle–dependent changes in phosphorylation on Thr-14 and Tyr-15. The trace number of nuclei added (20 to 50 per microliter) to monitor cell cycle progression was not sufficient to perturb the cell cycle progression owing to incomplete DNA replication (data not shown; Dasso and Newport, 1990). The results from this experiment are presented in Figures 5B and 5C. The control extract (lacking exogenous Cip, Cdk2, and cyclin E) initiated mitosis twice over the 130 min period of the experiment. This conclusion is based on the observed breakdown of nuclei, changes in Cdc2 phosphorylation, and peaks of histone H1 kinase activity that occurred at 45 and 100 min (data not shown). Extracts containing Cip alone failed to initiate mitosis over the same time course, as evidenced by the absence of nuclear envelope breakdown (Figure 5B), accumulation of phosphorylated Cdc2 (Figure 5C), and the absence of changes in H1 kinase activity (data not shown). By contrast, extracts containing Cip, GST–Cdk2, and GST–cyclin E, like control extracts, entered mitosis twice. In this extract, nuclear envelope breakdown (Figure 5B) and activation of Cdc2 kinase (Figure 5C) occurred at 50–60 min and again at 95–110 min. Based on these observations, it is clear that addition of Cdk2–cyclin E complexes to Cip-treated extracts efficiently restores cell cycle oscillation. Therefore, the inhibition of the initiation of mitosis that occurs in the presence of Cip is reversed when Cdk2–cyclin E kinase activity is restored to the extract.

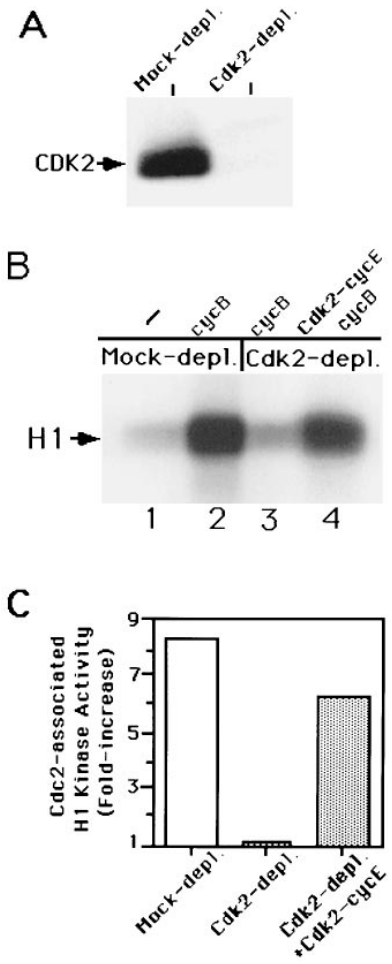

Figure 6. Resistance to Cdc2–Cyclin B Kinase Activation in Cdk2-Depleted Extracts

(A) Western blot analysis shows that Cdk2 protein is efficiently depleted from interphase extract. Affinity-purified rabbit immunoglobulin G (IgG) (obtained from Cappel, West Chester, PA) was used for mock depletions.

(B) Cdk2-depleted extracts were incubated in the absence or presence of 100 nM of both GST–Cdk2- and GST–cyclin E–purified proteins for 20 min at room temperature. GST–cyclin B fusion protein (35–70 nM final concentration) and sperm chromatin (50 nuclei per microliter) were added to the extracts for 60 min. Cdc2–cyclin complexes were immunoprecipitated and assayed for histone H1 kinase activity. Lane 1, mock-depleted extract alone; lane 2, mock-depleted extract with GST–cyclin B; lane 3, Cdk2-depleted extract with GST–cyclin B; lane 4, Cdk2-depleted extract with GST–cyclin B and 100 nM of both GST–Cdk2 and GST–cyclin E. Nuclei remained intact in Cdk2-depleted extracts (after 60 min), while control and Cdk2-rescued extracts entered mitosis at 45 min.

(C) Quantitation of histone H1 bands from gels. Cdc2-associated H1 kinase activity is expressed as fold increase relative to background control (21,090 cpm) of mock-depleted extract without GST–cyclin B.

Depletion of Cdk2–Cyclin E Blocks the Initiation of Mitosis in the Absence of Cip

The results above strongly suggest that Cdk2–cyclin E is an essential positive regulator for initiation of mitosis. Further, they predict that a reduction in Cdk2 kinase activity would, as in Cip-treated extracts, prevent the initiation of mitosis. To address this directly, we immunodepleted Cdk2 kinase from interphase extracts. A mock-depleted extract was prepared in parallel as a control. The efficiency of Cdk2 depletion was shown to be greater than 98%, as determined by Western blot analysis (Figure 6A). Either GST protein alone or 100 nM of both GST–Cdk2 and GST–cyclin E was added to a Cdk2-depleted extract and preincubated for 30 min at room temperature. To determine whether Cdk2-depleted extracts were competent to activate Cdc2–cyclin B, we added GST–cyclin B (35–70 nM) to both mock- and Cdk2-depleted extracts and then incubated the extracts an additional 60 min at room temperature. As a control for the specificity of the immunodepletion, GST–cyclin B was also added to a Cdk2-depleted extract that contained added active Cdk2–cyclin E kinase. Following this, Cdc2 was precipitated from a portion of each extract and assayed for H1 kinase activity. In the absence of GST–cyclin B, H1 kinase activity in mock-depleted extracts was very low (Figure 6B, lane 1). In mock-depleted extracts to which GST–cyclin B had been added, the H1 kinase activity was 8.3-fold higher than mock-depleted extracts lacking GST–cyclin B (Figures 6B, lanes 1 and 2, and 6C). In contrast, when GST–cyclin B was added to extracts depleted of Cdk2–cyclin E, Cdc2 remained inactive (Figures 6B, lane 3, and 6C). Importantly, restoration of Cdk2–cyclin E activity to Cdk2-depleted extracts rescued Cdc2 kinase activation (Figure 6B, lane 4) to levels similar to the mock-depleted

control (Figure 6C). Consistent with these observations, we found that Cdc2 protein associated with the added GST–cyclin B in Cdk2-depleted extracts was phosphorylated at both the Thr-14 and Tyr-15 negative regulation sites on Cdc2, based on its slower mobility on gels relative to unphosphorylated active Cdc2 from the mock-depleted and Cdk2–cyclin E–restored extracts (data not shown). These observations show that, even in the absence of Cip, the activation of Cdc2–cyclin B is strongly inhibited when Cdk2–cyclin E activity is absent from the extract. This result, together with the results presented above, strongly supports the proposal that Cdk2–cyclin E is a positive regulator essential for the initiation of mitosis in Xenopus egg extracts.

Discussion

That Cdk2 kinase plays an essential role in regulating the G1 to S phase transition during the cell cycle is well established. The results described in this report demonstrate that Cdk2 activity is also required for the G2 to M phase transition. In support of this, we have shown that addition of the potent Cdk2 inhibitor, $p21^{Cip1}$, to a cycling Xenopus extract blocks cell cycle progression into mitosis. That this inhibition of mitosis is due to Cdk2 inactivation is indicated by the following observations. First, the minimal concentration of Cip (75–100 nM) that completely inhibits Cdk2 kinase activity is sufficient to block entry into mitosis. Importantly, the Cip-induced arrest is reversed by restoring Cdk2–cyclin E kinase activity. Under these conditions, cycling extracts enter and exit mitosis at a rate similar to control extracts (Figures 5B and 5C). Lastly, we have shown more directly that Cdk2 complexes are essential for initiation of mitosis. Thus, extracts quantitatively depleted of Cdk2, like Cip-treated extracts, fail to initiate mitosis when exogenous cyclin B is added. Quite interestingly, this requirement for Cdk2 in promoting the activation of Cdc2 in not dependent on DNA replication or transcription. This suggests a biochemical linkage between Cdk2 and Cdc2 activities. Taken together, these observations strongly support the claim that Cdk2 kinase positively regulates the biochemical pathways that control the initiation of mitosis.

In budding yeast, precedence exists for a regulatory interaction between distinct Cdk–cyclin kinases in controlling the sequential phases of the cell cycle. Specifically, it has been shown that the proteolytic system of mitotic Clb cyclins, which is activated during mitosis, remains active during the subsequent G1 period until Cln–Cdc28 kinase complexes accumulate during late G1 phase (Amon et al., 1994). Further, Cln–Cdc28 activation in late G1 phase is required for the sequential expression and activation of Clb–Cdc28 kinase complexes during S and G2/M phases (Schwob et al., 1994). Thus, in budding yeast the functional equivalent of Xenopus Cdk2 may positively regulate the equivalent of Xenopus Cdc2.

However, unlike budding yeast, we have shown that active Cdk2 kinase is required for neither the synthesis nor the association of newly synthesized mitotic cyclins with Cdc2 (Figure 2C). Instead, in the absence of Cdk2 kinase activity, Cdc2–cyclin B complexes accumulate and remain inactive, apparently owing to phosphorylation of the Thr-14 and Tyr-15 negative regulatory sites on Cdc2. Moreover, CAK kinase activity is unlikely to be affected by lack of Cdk2 kinase activity in an interphase egg extract. Supporting this, it was recently shown that $p21^{Cip1}$ does not interact directly with CAK kinase (Harper et al., 1995). More directly, inactive Cdc2–cyclin complexes immunoprecipitated from egg extracts that lack Cdk2 kinase activity are rapidly activated when treated with purified human GST–Cdc25B phosphatase (T. M. G. and J. W. N., unpublished data). Thus, Cdc2–cyclin complexes are phosphorylated by CAK kinase at Thr-161, and it seems likely that Cdc2 activity is blocked, at least in part, by phosphorylations at Thr-14/Tyr-15. Finally, we have observed that addition of at least 8-fold higher concentrations of GST–cyclin B protein (240 nM) in Cdk2-depleted or Cip-treated extracts readily activates Cdc2 kinase. This observation is consistent with a previous observation made by Fang and Newport (1991) that large amounts of cyclin B added to a Cdk2-depleted extract readily activates mitosis. It is likely that, at these very high concentrations, all checkpoint controls regulating Cdc2 activation are ineffective owing to the rapid activation of the Cdc2 autocatalytic activation loop. Nevertheless, our results demonstrate that at physiological levels of GST–cyclin B (35–70 nM), Cdc2–cyclin B activation is strictly dependent on Cdk2 kinase activity.

Because Cdc2 kinase is essential for initiation of mitosis, our results suggest that Cdk2 positively regulates mitosis at a point upstream from Cdc2 activation. Mechanistically, this could occur if Cdk2 either down-regulated the kinases that inactivate Cdc2 or up-regulated the Cdc25 phosphatases that activate Cdc2. Small changes in either of these two activities would greatly reduce the threshold concentration at which the extremely sensitive autocatalytic loop that activates Cdc2 would begin to function. With respect to this possibility, it is interesting to note that in the absence of Cip, Cdc25 becomes partially phosphorylated well before (30–60 min) the onset of mitosis (90 min), while in the presence of Cip, this partial phosphorylation is completely absent (Figure 2B). Because phosphorylation is integral to the activation of Cdc25 (Hoffmann et al., 1993; Kumagai and Dunphy, 1992; Izumi and Maller, 1993), this partial phosphorylation could serve to increase Cdc25 activity. Alternatively, if Cdc2 activity is also regulated by the association of currently unidentified regulatory factors (Kumagai and Dunphy, 1995), Cdk2 could modulate the interaction of such factors with Cdc2 complexes. A detailed molecular understanding of how Cdk2 positively regulates initiation of mitosis will require further experimentation.

Coordinating S Phase and Mitosis by Cdk2 Kinase

In evaluating why direct regulation of Cdc2 by Cdk2 would confer a selective advantage to cells, it is important to consider the regulatory network that ensures that S phase precedes mitosis. Figure 7 is a schematic

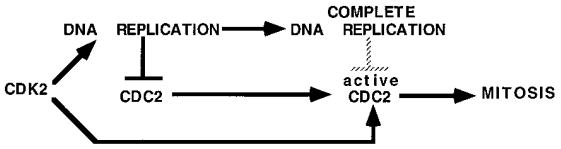

Figure 7. Schematic Illustration Summarizing How S Phase and Mitosis May Be Coordinated by Cdk2 Kinase

See Discussion for explanation. The black and white striped symbol represents the attenuation of negative feedback signals that occurs upon the completion of DNA replication.

summary of how Cdk2 kinase may coordinate the sequential regulation of S phase followed by mitosis. During the cell cycle, active Cdk2 kinase is essential for initiating DNA replication at S phase. At this time, ongoing DNA replication activates a pathway that negatively regulates Cdc2 (Dasso and Newport, 1990). The activity of this regulatory pathway inhibits Cdc2 until all DNA has been replicated, therefore preventing premature mitosis before the completion of S phase. Upon the completion of DNA replication, the replication-dependent pathway that inhibits Cdc2 is attenuated. The data in this report show that, in the absence of DNA replication, Cdk2 kinase activity is absolutely required for positively regulating Cdc2–cyclin B kinase. Thus, after the completion of DNA replication, Cdk2 kinase continues to drive the cell cycle forward by positively regulating Cdc2–cyclin B kinase, an activity essential for the initiation of mitosis.

Does Cdk2-Dependent Regulation of Entry into Mitosis Occur in Somatic Cells?

During the short embryonic cell cycles that occur during early Xenopus development, Cdk2–cyclin E kinase levels remain high throughout the cell cycle (Fang and Newport, 1991; Rempel et al., 1995; Howe and Newport, submitted). Moreover, during this period of development, Cdk2–cyclin E is sufficient to initiate and drive genomic DNA replication to completion during each cell cycle. At this time in development, cyclin A is primarily associated with Cdc2 (Minshull et al., 1989; Rempel et al., 1995) and is not essential for DNA replication (Fang and Newport, 1991). This situation differs significantly from that which occurs during the cell cycle in somatic cells. In somatic cells, Cdk2–cyclin E activity accumulates in late G1, peaks at the G1–S boundary, and then decays rapidly, such that little if any activity is present by mid-S phase (Dulic et al., 1992; Koff et al., 1992). Clearly, the absence of Cdk2–cyclin E activity by late S phase in somatic cells precludes this complex from playing a positive role in regulating the onset of mitosis. Rather, it is likely that this function is carried out by Cdk2–cyclin A complexes. Unlike Cdk2–cyclin E complexes, Cdk2–cyclin A activity first appears at late G1 phase and continues to accumulate during both S and G2 phases of the cell cycle (Pagano et al., 1992, 1993; Rosenblatt et al., 1992; Tsai et al., 1993), before being degraded at mitosis. Thus, in somatic cells, we believe it is quite likely that Cdk2–cyclin A complexes, like Cdk2–cyclin E in Xenopus extracts, may play an essential role in positively regulating the initiation of mitosis.

In summary, our results in Xenopus egg extracts strongly argue that coordinated execution of S phase and mitosis involves a regulatory pathway that directly links Cdk2 and Cdc2 activities. Linkage between Cdk kinase family members, in combination with checkpoint controls, would establish a precise and tight control system for directing cell cycle progression from the G1–S transition to mitosis.

Experimental Procedures

Preparation of Xenopus Egg Extracts

Interphase extracts and demembraned sperm chromatin were prepared as previously described (Smythe and Newport, 1991). CSF extracts (arrested in mitosis) were prepared from unfertilized Xenopus eggs according to the protocol of Murray (1991). Cycling extracts that oscillate between S phase and mitosis were prepared from electrically activated eggs as described by Murray and Kirschner (1989). Nuclear envelope assembly and disassembly was observed by phase-contrast and UV fluorescence microscopy (Dasso and Newport, 1990).

Isolation of Fusion Proteins

A plasmid encoding Cip protein as a GST fusion was provided by Dr. S. Elledge. Plasmid DNAs encoding histidine-tagged Cip (pET-His-Cip) and GST fused to Xenopus cyclin E1 (pGST-XeCycE1) proteins were provided by Dr. T. Hunt. A full-length Xenopus *Cdk2* cDNA fragment containing a NcoI and blunt-ended EcoRI ends was subcloned in the pGEX-KG vector at its NcoI site (pGST-Cdk2). A plasmid encoding a nondegradable sea urchin cyclin B1 (missing 13 N-terminal amino acids) fused to GST was provided by M. Solomon (Yale University). All plasmid DNAs were transformed into strain BL21(DE3) pLysS, and recombinant fusion proteins were expressed as described previously (Connell-Crowley et al., 1993) with induction at 25°C. Recombinant GST fusion proteins were isolated from bacterial lysates by affinity chromatography on glutathione–Sepharose beads (Pharmacia, Piscataway, NJ) as previously described (Solomon et al., 1990). Histidine-tagged Cip protein was affinity purified from bacterial lysates on His–Bind resin (Novagen, Madison, WI) according to the instructions of the manufacturer. Fractions containing recombinant proteins were concentrated in Centricon 10 or 30 concentrators (Amicon, Beverly, MA) and buffer-exchanged in XB buffer (100 mM KCl, 50 mM sucrose, 10 mM HEPES, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ [pH 7.7] with KOH). The final protein concentration was determined by Coomassie staining. Titration of GST–Cip or His-tagged Cip preparations demonstrated that 75–100 nM concentrations resulted in >95% inhibition of Cdk2 kinase activity and DNA replication in extracts. Cip concentrations as high as 300 nM did not inhibit active Cdc2 kinase.

Antibodies

Affinity-purified Xenopus Cdc25 antibodies were provided by A. Kumagai (Dunphy laboratory), Xenopus cyclin B2 antibodies were provided by T. Lee (Kirschner laboratory), and Xenopus cyclin A1 antibodies were provided by J. Maller. The polyclonal antibodies for detecting Xenopus Ckd2 and Cdc2 proteins were prepared as previously described (Fang and Newport, 1991). Xenopus cyclin E antibody was raised in rabbits against an N-terminal cyclin E1 peptide (SVRSRKRKADVA) by Research Genetics (Huntsville, AL) and affinity purified as described by Howe and Newport (submitted).

Precipitations, Immunodepletions, and Western Blot Analysis

Cdk complexes bound to GST fusion proteins (Cip or sea urchin cyclin B) were isolated from Xenopus egg extracts on glutathione–Sepharose beads (Pharmacia) or p13–Sepharose beads, as previously described (Smythe and Newport, 1992). Immunoprecipitation of Cdc2, Cdk2, or cyclin E was carried out as described by Fang and Newport (1991). Immunodepletion of Cdk2 from interphase extracts was performed as described (Fang and Newport, 1991).

For protein analysis, we employed standard immunoblotting procedures (Harlow and Lane, 1988) with appropriate antibodies and ECL reagents (Amersham, Chicago, IL). For assaying Cdc2 protein mobility shifts due to Thr-14 and Tyr-15 phosphorylation, protein

samples were resolved electrophoretically on 25 cm 10% SDS–polyacrylamide protein gels.

Histone H1 Kinase Assays

Histone H1 kinase assays of extracts or immunoprecipitated complexes were performed as described previously (Fang and Newport, 1991). All samples were electrophoresed on 10% SDS–polyacrylamide gels, Coomassie blue stained (to visualize histone H1), dried, and exposed to X-ray film to detect phosphorylation of H1 substrate. In some cases, ^{32}P incorporation was quantitated by scintillation counting of excised histone H1 gel slices.

Acknowledgments

We thank Steve Elledge, James Maller, Tina Lees (Kirschner laboratory), Tim Hunt, and Bill Dunphy for providing reagents and Rajeev Soni (Paul Russell laboratory) for purified active human GST–Cdc25B protein. We are grateful for all helpful discussions and advice from members of the Forbes and Newport laboratories. Many thanks go to Dr. Sally Kornbluth (Duke University) for valuable help during the early stages of this work. Finally, we especially thank Drs. Sarah Guadagno and Hong Yan for their critical reading of the manuscript and helpful suggestions. This work was supported by National Institutes of Health (NIH) grant GM 33523-07 to J. W. N. and an NIH postdoctoral fellowship (HD07721-03) to T. M. G.

Received September 12, 1995; revised November 8, 1995.

References

Amon, A., Irniger, S., and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell *77*, 1037–1050.

Atherton-Fessler, S., Liu, F., Gabreilli, B., Lee, M.S., Peng, C.Y., and Piwnica-Worms, H. (1994). Cell cycle regulation of the $p34^{cdc2}$ inhibitory kinases. Mol. Biol. Cell *5*, 989–1001.

Connell-Crowley, L., Solomon, M.J., Wei, N., and Harper, J.W. (1993). Phosphorylation independent activation of human cyclin-dependent kinase 2 by cyclin A *in vitro*. Mol. Biol. Cell *4*, 79–92.

Dasso, M., and Newport, J.W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. Cell *61*, 811–823.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell *56*, 829–838.

Dulic, V., Lees, E., and Reed, S.I. (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. Science *257*, 1958–1961.

Dunphy, W.G., and Kumagai, A. (1991). The cdc25 protein contains an intrinsic phosphatase activity. Cell *67*, 189–196.

Dunphy, W.G., Brizuela, L., Beach, D., and Newport, J. (1988). The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell *54*, 423–431.

Enoch, T., Carr, A.M., and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to the completion of DNA replication. Genes Dev. *6*, 2035–2046.

Fang, F., and Newport, J. (1991). Evidence that the G1–S and G2–M transitions are controlled by different Cdc2 proteins in higher eukaryotes. Cell *66*, 731–742.

Featherstone, C., and Russell, P. (1991). Fission yeast $p107^{wee1}$ mitotic inhibitor is a tyrosine/serine kinase. Nature *349*, 808–811.

Fesquet, D., Labbe, J.C., Derancourt, J., Capony, J.P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M., and Cavadore, J.C. (1993). The M015 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKS) through phosphorylation of Thr161 and its homologues. EMBO J. *12*, 3111–3121.

Fisher, R.P., and Morgan, D.O. (1994). A novel cyclin associates with M015/Cdk7 to form the CDK-activating kinase. Cell *78*, 713–724.

Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene *cdc2*+. Cell *54*, 433–439.

Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991). Cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell *67*, 197–211.

Gould, K., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast *cdc2*+ protein kinase regulates entry into mitosis. Nature *342*, 39–45.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L-H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., Fox, M.P., and Wei, N. (1995). Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell *6*, 387–400.

Hartwell, L., and Weinert, T. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629–634.

Heichman, K.A., and Roberts, J.M. (1994). Rules to replicate by. Cell *79*, 556–562.

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. *12*, 53–63.

Hunter, T., and Pines, J. (1994). Cyclins and cancer II: cyclin D and Cdk inhibitors come of age. Cell *79*, 573–582.

Izumi, T., and Maller, J.L. (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. Mol. Biol. Cell *4*, 1337–1350.

Izumi, T., Walker, D.H., and Maller, J.L. (1992). Periodic changes in phosphorylation of the *Xenopus* cdc25 phosphatase regulate its activity. Mol. Biol. Cell *3*, 927–939.

Jackson, P.K., Chevalier, S., Philippe, M., and Kirschner, M.W. (1995). Early events in DNA replication require cyclin E and are blocked by $p21^{CIP1}$. J. Cell Biol. *130*, 755–769.

Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Philippe, M., and Roberts, J. (1991). Human cyclin E, a new cyclin that interacts with two members of the *CDC2* gene family. Cell *66*, 1217–1228.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R., and Roberts, J.M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science *257*, 1689–1694.

Kornbluth, S., Sebastian, B., Hunter, T., and Newport, J. (1994). Membrane localization of the kinase which phosphorylates $p34^{cdc2}$ on threonine 14. Mol. Biol. Cell *5*, 273–282.

Kumagai, A., and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. Cell *70*, 139–151.

Kumagai, A., and Dunphy, W.G. (1995). Control of the cdc2/cyclin B complex in *Xenopus* egg extracts arrested at a G2/M checkpoint with DNA synthesis inhibitors. Mol. Biol. Cell *6*, 199–213.

Lew, D.J., Dulic, V., and Reed, S.I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell *66*, 1197–1206.

Matsuoka, M., Kato, J.-y., Fisher, R.P., Morgan, D.O., and Sherr, C.J. (1994). Activation of cyclin-dependent kinase-4 (CDK4) by mouse M015-associated kinase. Mol. Cell. Biol. *14*, 7265–7275.

Minshull, J., Blow, J., and Hunt, T. (1989). Translation of cyclin mRNA is necessary for extracts of activated Xenopus eggs to enter mitosis. Cell *56*, 947–956.

Minshull, J., Golsteyn, R., Hill, C.S., and Hunt, T. (1990). The A- and B-type cyclin associated cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. EMBO J. *9*, 2865–2875.

Mueller, P.A., Coleman, T.R., Kumagai, A., and Dunphy, W.G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates cdc2 on both threonine-14 and tyrosine-15. Science *270*, 86–90.

Murray, A. (1991). Cell cycle extracts. Meth. Cell Biol. *36*, 581–607.

Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275–280.

Murray, A.W., Solomon, M.J., and Kirschner, M.W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature *339*, 280–286.

Nurse, P. (1994). Ordering S phase and M phase in the cell cycle. Cell *79*, 547–550.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO J. *11*, 961–971.

Pagano, M., Pepperkok, R., Lukas, J., Baldin, V., Ansorge, W., Bartek, J., and Draetta, G. (1993). Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. J. Cell Biol. *121*, 101–111.

Pardee, A.B. (1989). G1 events and regulation of cell proliferation. Science *246*, 603–608.

Poon, R.Y., Yamashita, K., Adamczewski, J.P., Hunt, T., and Shuttleworth, J. (1993). The cdc2-related protein $p40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $p33^{cdk2}$ and $p34^{cdc2}$. EMBO J. *12*, 3123–3132.

Rempel, R.E., Sleight, S.B., and Maller, J.L. (1995). Maternal *Xenopus* cdk2-cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G1 phase. J. Biol. Chem. *270*, 6843–6855.

Rosenblatt, J., Gu, Y., and Morgan, D.O. (1992). Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. Proc. Natl. Acad. Sci. USA *89*, 2824–2828.

Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by *wee1*+, a gene encoding a protein kinase homolog. Cell *49*, 559–567.

Schwob, E., Böhm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40*SIC1* controls the G1 to S transition in S. cerevisiae. Cell *79*, 233–244.

Sherr, C.J. (1994). G1 phase progression: cycling on cue. Cell *79*, 551–555.

Smythe, C., and Newport, J. (1991). Systems for the study of nuclear assembly, DNA replication, and nuclear breakdown in *Xenopus laevis* egg extracts. Meth. Cell Biol. *35*, 449–468.

Smythe, C., and Newport, J. (1992). Coupling of mitosis to the completion of S phase in Xenopus occurs via modulation of the tyrosine kinase that phosphorylates p34*cdc2*. Cell *68*, 787–797.

Solomon, M.J., Glotzer, M., Lee, T.H., Philippe, M., and Kirschner, M.W. (1990). Cyclin activation of p34*cdc2*. Cell *63*, 1013–1024.

Solomon, M.J., Harper, J.W., and Shuttleworth, J. (1993). CAK, the $p34^{cdc2}$ activating kinase, contains a protein identical or closely related to $p40^{MO15}$. EMBO J. *12*, 3133–3142.

Strausfeld, U.P., Howell, M., Rempel, R., Maller, J.L., Hunt, T., and Blow, J.J. (1994). $p2I^{Cip1}$ blocks the initiation of DNA replication in *Xenopus* extracts and can be rescued by cyclins A and E, but not B. Curr. Biol. *4*, 876–883.

Tsai, L., Harlow, E., and Meyerson, M. (1991). Isolation of the human *cdk2* gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. Nature *353*, 174–177.

Tsai, L., Lees, E., Faha, B., Harlow, E., and Riabowol, K. (1993). The cdk2 kinase is required for the G1-to-S transition in mammalian cells. Oncogene *8*, 1593–1602.

van der Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science *262*, 2050–2054.

Yan, H., and Newport, J.W. (1995). An analysis of the regulation of DNA synthesis by cdk2, Cip1, and licensing factor. J. Cell Biol. *129*, 1–15.